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MOUSE MODELS FOR ASSESSING PROTEIN IMMUNOGENICITY: LESSONS AND CHALLENGES

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Abstract

The success of clinical and commercial therapeutic proteins is rapidly increasing, but their potential immunogenicity is an ongoing concern. Most of the studies that have been conducted over the past few years to examine the importance of various product-related attributes (in particular several types of aggregates and particles) and treatment regimen (such as dose, dosing schedule and route of administration) in the development of unwanted immune responses have utilized one of a variety of mouse models. In this review we discuss the utility and drawbacks of different mouse models that have been used for this purpose. Moreover, we summarize the lessons these models have taught us and some of the challenges they present. Finally, we provide recommendations for future research utilizing mouse models to improve our understanding of critical factors that may contribute to protein immunogenicity.

Keywords

Protein aggregation; immunogenicity; in vivo models

INTRODUCTION

Therapeutic proteins such as monoclonal antibodies (mAbs), cytokines, blood factors, growth factors and hormones have become an important class of drugs.¹ Despite their

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clinical and commercial successes, however, an ongoing concern with the use of therapeutic proteins is their potential immunogenicity, i.e., some patients receiving these drugs produce anti-drug antibodies (ADAs) which may be associated with various potential clinical consequences.² Most protein therapeutics are immunogenic in at least some patients³; with some protein products (e.g., interferon beta⁴) greater than 30–50% of patients experience immune responses that can result in reduced efficacy. Protein immunogenicity depends on multiple factors that are related not only to the product,^{5–12} but also to the patient (e.g., disease state) and the treatment regimen.^{5–7} Unfortunately, there are still major gaps in our understanding with respect to the relative importance of each of these factors and how they mutually influence each other. In an attempt to overcome these shortcomings, several different preclinical *in silico* models^{13–15}, *in vitro* models^{13–19} and *in vivo* models^{14,15,20–22} have been developed by a number of groups to study protein immunogenicity, and especially the relative contributions of product-related factors. Although at present none of these approaches can completely simulate the course of development of unwanted immune responses in the clinic, the currently available preclinical models are valuable tools for investigating various product-related attributes or different molecule variants. For the purposes of the discussion in this commentary product-related attributes include different types of protein aggregates and fragments, and chemical degradation products. The effect of the presence of foreign materials (e.g., glass, stainless steel, silicone oil) on protein immunogenicity has been studied as well. *In vivo* models can also be used to some extent for evaluating the relative impact of administration-related factors. Animal models have been particularly popular, because, unlike *in silico* and *in vitro* models, they provide an intact immune system. Thus, though they are different than humans, murine models present the only option to study these, as clinical studies on the impact of product-related attributes cannot be performed for ethical reasons. The formation of ADAs, the endpoint of the humoral response, can be directly measured in the *in vivo* models. In contrast, *in vitro* assays usually rely on indirect measurements, such as cytokine release or immune cell stimulation which represent a single step in the complex immune response. Although historically a variety of animal models has been used to study immunogenicity of proteins, most of the studies that have been conducted over the past few years to examine the potential importance of various product-related attributes (as defined above) have been conducted in murine models.^{21–29} Others have used these *in vivo* models (see Table 1 for an overview) to study the effects of route of delivery,^{26,30} as well as the potential molecular mechanisms behind immune responses that could help mitigating such reactions in the future.³¹ The mouse strains used in these studies were very diverse. For instance, mice capable of forming large antibody repertoires and a diverse response of human antibodies following immunization were used to evaluate ADA responses to attributes of a human monoclonal IgG1.^{22,23} Similarly, a human immunoglobulin G2 (IgG2)-tolerant and immune-competent heterozygous mouse model (Xeno-het) derived from a C57BL/6J wild-type strain expressed both mouse and human immunoglobulin G (IgG) genes, resulting in B-cells expressing human and mouse IgG, and secretion of human and mouse immunoglobulins into serum.²¹ The cross breeding with the C57BL/6J strain ensured robust B cell signaling and diverse repertoires.

Some groups have also utilized the nude and immune competent BALB/c mouse strains as they are more suitable to distinguish the T-cell dependent and T-cell independent B-cell responses that can be distinguished through the secreted IgG isoforms.³² Some clear trends have begun to emerge on the utility and drawbacks of different murine models as well as on the product quality attributes that elicit the greatest immune response in these model systems. Below we summarize the lessons we have learned from these studies, as well as some of the challenges that they present.

CHOICE OF MOUSE MODEL

Wild-type mice

Since the 1960s, there have been numerous studies in which the immunostimulatory effects of aggregates and particles formed from non-murine proteins have been characterized in wild-type mice. Some studies suggest that in the absence of aggregates and particles, nominally foreign proteins were tolerated immunologically. For instance, Dresser reported that formulations of bovine γ globulin were not immunogenic in mice if they were treated by centrifugation to remove insoluble particles prior to injection.³³ More recently, Fradkin et al. showed that the immunogenicity of recombinant human growth hormone (rhGH) in mice decreased significantly when aggregate and particle levels were reduced by high-pressure treatment.³⁴ However, in some studies in wild-type mice, the immunogenicity induced by the native recombinant human therapeutic protein is so high that it is difficult to observe any additional effects from factors (e.g., aggregation or chemical degradation) that are believed to increase the risk of immunogenicity.^{21,35–41} Nevertheless, wild-type mice might be useful for studying effects of therapeutic replacement proteins that are used to treat patients who are unable to produce certain enzymes, hormones and clotting factors; in these patients the therapeutic human proteins might be perceived by the immune system as foreign.

A more recent approach utilizes murine variants of human proteins, as has been done for growth hormone,^{26,32} interferon- β ⁴² and monoclonal IgG.^{27,28,43} These models have been useful for investigations on the relative impacts of product-related factors on immunogenicity, as well as immune mechanisms involved in the breaking of immune tolerance (for a description of the concept of immune tolerance, see Goodnow et al.⁴⁴). A practical advantage of this approach is that the mice in principle are suitable for testing the immunogenicity of any murine protein. In addition, commercially available mouse strains can be used and their breeding is straightforward.

Transgenic mice

Transgenic mice have been used in several studies of immune responses to therapeutic proteins. Why should one take the trouble to generate transgenic mice that express human proteins of therapeutic interest? The reason for this is that in many cases, patients are receiving fully human or humanized proteins to which they have a high level of immune tolerance, and with the transgenic mouse the same human protein can be used for immunogenicity studies. The mouse model used should mimic a state of tolerance to self-protein that has to be broken to generate ADAs, because the mechanism of immunogenicity

may differ from that of a classical response against a foreign antigen, such as in vaccinations.

Transgenic models have been established for investigating the immunogenicity of recombinant human therapeutic proteins, such as recombinant human interferon alfa (rhIFN α),⁴⁵ interferon beta (rhIFN β),^{35,38} rhGH,³⁴ insulin^{25,46} and monoclonal IgG.^{21–23,47} The major advantage of this approach is that these animals can be used to study the effects of different factors that may lead to immunogenicity of recombinant human protein drugs for which they are immune tolerant within an intact immune system, just like many patients at the start of a therapy. Disadvantages of this approach (in addition to the differences between humans and animals such as physiology and dosing regimen that are common to all animal model systems) are that the mice can be used for only one type of protein, the expressed human protein may exert unpredictable biological effects in mice, and expression levels may not match those in humans. Also, construction of the transgenic mouse strains is time consuming and the breeding may be cumbersome, depending on the model. Moreover, one should keep in mind that these mice still have a murine immune system and there is no representation of relevant human HLA and antigen presenters, which would be needed to evaluate sequence-based as well as long-term adaptive phase associated immunogenicity risk.

Critical factors that affect the utility of mouse models

Several factors are crucial for the successful application of a mouse model. The choice of the mouse strain is of great importance, as the immune response is strongly dependent on the strain.^{38,43,46} For instance, BALB/c and C57BL/6 mice differ in their immunological and functional architecture and their sensitivity to antigenic stimuli. In BALB/c mice, T-cell functionality and the cytokine release capability is much higher than in C57BL/6 mice, making BALB/c mice better models for studying adaptive phase responses. Similarly, C57BL/6 mice are characterized by higher cytostatic activity of splenic NK cells, making them better models to investigate innate phase responses.⁴⁸ Additionally, C57BL/6 and BALB/c mice differ in their responsiveness to antigen induced pro-inflammatory and immune suppressive cytokines.⁴⁹

Another important factor is the timing for the withdrawal of blood samples from mice. Because detection of anti-drug antibodies in the presence of circulating drug may be difficult, the optimal sampling schedule may be influenced by the serum half-life of the therapeutic protein in the mice, the applied dose and the dosing schedule. The sampling schedule may also depend on the type of immune response that is anticipated. For example, detection of long-term memory responses may require extended periods of time between doses.

Determining a suitable administration/sampling schedule can be very time-consuming, depending on the protein and the analytical assays used to detect immunogenicity in the presence of circulating drug.⁵⁰ For instance, a dose of 5 μ g/injection and 15 injections over a period of three weeks for hIFN α and hIFN β transgenic mice, following the protocol originally published by Braun et al.,⁴⁵ was successful in generating ADAs. However, for monoclonal IgG and insulin this administration protocol did not result in measurable ADA

levels even in the (non-immune-tolerant) wild-type mice, and new protocols had to be developed in order to obtain satisfactory results.^{23,25} It is worth noting that dosing and administration schemes used for these studies in general are not meant to translate directly to the dosing scheme in humans.

Even in transgenic strains, there are several challenges to their use to mimic potential immune responses in humans. Although the mice may be tolerant for the human protein, the predominant background of the immune response is murine, which may limit their ability to predict immunogenicity in humans. For instance, the HLA restriction to a single allele results in a homozygous genotype, which is very rare in humans, and these mice also often lack the ability to mount a robust polyclonal and affinity-matured immune response.⁵¹ Another important consideration when interpreting results from these model systems is that these are inbred populations with little overall genetic variability. This decreases the variability of the background against which the results are obtained and could make them easier to interpret, but it also implies that the differences in genetic makeup, age, concurrent treatments and other variables that are seen with human populations in the clinic will be missed.

EFFECTS OF PRODUCT-RELATED FACTORS ON IMMUNOGENICITY

There are several product-specific factors that may impact the immunogenicity of a therapeutic protein. These include potential immunogenic T-cell epitope content in the protein's sequence, presence of host cell proteins and/or DNA, type and amount of degradants and impurities (such as aggregates and particulates) present, glycosylation patterns, immunomodulatory activity of the therapeutic protein, protein conformation, and type and extent of chemical modifications, to name a few. For example, the rhIFN β products Avonex, Rebif and Betaferon have shown varying levels of immunogenicity in the clinic, with Betaferon in general being the most immunogenic.^{8,52,53} However, in addition to the product itself, the dose, dosing frequency and route of administration are different for these products, and different assays were used to assess immunogenicity. In a head-to-head comparison (same dose, dosing schedule and administration route) in an immune-tolerant mouse model, Betaferon was clearly the most immunogenic product of the three, breaking immune tolerance in all animals to which it was administered.³⁸ This effect might be related to Betaferon's relatively high aggregate content,^{54,55} but other differences (e.g., sequence, glycosylation pattern, chemical degradation, excipients) may also play a role. Altogether, these studies demonstrate that differences in product quality may affect immunogenicity, highlighting the importance of understanding the role of storage, handling, administration, process, and formulation conditions during commercial manufacturing and use of therapeutic proteins to minimize impact on product quality.

The presence of aggregates has been highlighted as an important product-related risk factor for immunogenicity.^{6,31,56–59} Hence, many of the studies with immune-tolerant mouse models so far have focused on probing the role of aggregates in protein immunogenicity. The collective results from immune-tolerant mouse studies have shown that aggregates and particles can lead to the breaking of tolerance and enhanced immunogenicity, but also that not all types of aggregates or particles are equally immunogenic and that some aggregates

and particles appear to not be immunogenic at all. Instead, the immunogenicity of protein aggregates and particles seems highly dependent on their characteristics (including chemical modifications) which in turn depend on the way they are created.^{23,34,60,61}

Protein aggregates containing chemically modified protein are often immunogenic

Aggregates created by metal-catalyzed oxidation have consistently shown high immunogenicity compared to other aggregates and unstressed protein, as demonstrated for rhIFN α -2b,^{36,62} rhIFN β -1a,⁴⁰ monoclonal IgG²³ and insulin.²⁵ Significant oxidation has been shown to induce, besides aggregation, some conformational changes.^{23,25,40,60,62} Moreover, all of these aggregates showed some oxidation of histidine^{61,63} and the aromatic amino acid residues.^{63,64} Hydrogen peroxide-mediated oxidation,^{21,40} stirring⁶¹ and UV-light exposure^{22,24,28} have also been used to generate protein aggregates which have enhanced immunogenicity in the murine model system tested compared to that of the protein before stress treatment. It is important to note that all of the chemical modifications described above were induced by treatments that increased the amount of such modifications to levels that are not representative of what typically would be seen in the clinic. Several studies have shown that some chemically modified monomeric proteins have not been immunogenic,^{21,22,62} suggesting that certain chemical changes may not be sufficient to break immune tolerance.

Protein conformation possibly affects aggregate immunogenicity

Compared to unstressed protein, rhIFN α -2b aggregates³⁶ and monoclonal IgG1 aggregates²³ created by heat and/or pH stress showed enhanced immunogenicity, albeit to a lesser extent than aggregates of the corresponding protein created by (metal-catalyzed) oxidation. These aggregates were shown to be composed of protein molecules that appear to be structurally perturbed but not fully denatured (retaining some folded structure).^{23,36} Stirring-induced IgG2 aggregates induced a low and transient response in Xeno-het mice²¹ and also retained a significant degree of their folded structure.⁶⁰ Aggregates of recombinant murine growth hormone (rmGH) formed by freeze-thawing or agitation contained protein molecules with native-like secondary structure and were immunogenic in wild-type mice.³² Interestingly, the antibody isotypes produced in response to these aggregates were different and somewhat lower in titers than those produced when rmGH was adsorbed to glass or aluminum hydroxide microparticles, where the protein structure was even more native-like.³² In line with these findings, native-like aggregates of recombinant factor VIII were reported to be more immunogenic than the native protein in hemophilia A mice.⁶⁵ In contrast, aggregates consisting of largely denatured protein created by heating^{28,62,65} or guanidine treatment^{40, 62} were not able to break immune tolerance to the native protein, suggesting that some residual 'native-like' structure is needed for breaking immune tolerance to the native protein. These results suggest that aggregates composed of protein with a somewhat perturbed structure may be more immunogenic (or perhaps produce immune responses that are more cross-reactive with native protein molecules) than aggregates consisting of either native-like or denatured protein. Alternatively, these results might indicate that the modification of B cell epitopes, being predominantly conformational, is not sufficient to break tolerance. Another study showed that IgG1 aggregates composed of largely native-like protein molecules created by freeze-thaw or shaking stress were also not immunogenic in mice.²³

Aggregate size may affect immunogenicity

Aggregates can differ in size by several orders of magnitude.⁶⁶ Aggregates as small as dimers produced by severe UV-light stress of an IgG1 induced only a very minor ADA response in transgenic mice,²² with titers being significantly lower than those induced in response to oligomers generated by the same stress method. Similarly-sized aggregates produced by process-related conditions and low pH, as well as covalently modified monomers, were not immunogenic.²² A few other publications suggest that larger aggregates tend to be more immunogenic than smaller ones with otherwise similar properties. For instance, in a study that used murine IgG2c, a fraction containing micron-sized aggregates (containing conformationally perturbed protein) induced by UV-light exposure was more immunogenic than a fraction containing soluble oligomers.²⁸ In the same study, subvisible aggregates created by mechanical stress also showed enhanced immunogenicity, albeit to a lower extent.

The results of studies on the impact of subvisible protein aggregates on immunogenicity are somewhat contradictory. On the one hand, rhIFN β -1a immunogenicity tended to be enhanced in samples containing elevated levels of micron-sized aggregates, rather than being dependent on total aggregate content.³⁹ On the other hand, Filipe et al. observed that the immunogenicity of differently stressed monoclonal IgG samples did not correlate with the concentrations of micron-sized or submicron-sized aggregates in these samples,²³ implying that factors other than subvisible particle concentration played a more important role.

In conclusion, aggregate size may have an impact on protein immunogenicity. However, the results from different studies are somewhat conflicting, most likely because aggregate attributes other than size (such as chemical and physical modifications), which may depend on the treatment protocol, and the molecule being tested, are at least as important. Furthermore, current available technology used to fractionate, purify, stabilize and deliver aggregates and particles in narrow, well-defined size ranges is limited.⁶⁷ Even when aggregates are fractionated, they may be unstable, and any changes in aggregate distributions that may occur upon administration are unknown. The reversibility/dissociability of aggregates under *in vivo* conditions could be important in this regard.

Could non-proteinaceous particles play a role in modulating immunogenicity?

Non-proteinaceous particles may be a risk factor especially if proteins are prone to adsorb to them. A number of experiments have been carried out using suspensions of various non-proteinaceous particles (see below) to evaluate their effects on potential immune reactions. One common caveat of such model studies is that the types of the particles used (nature, amount, size-distribution and homogeneity) are often not representative of the non-proteinaceous particles present in therapeutic products. It remains to be seen to what extent these limitations impact the relevance of such studies.

RhIFN β -1a, when adsorbed to 14- μ m stainless steel particles, showed enhanced immunogenicity as compared to native, unadsorbed rhIFN β -1a.⁶⁸ Although rhIFN β -1a also adsorbed to 0.2- μ m carboxylated polystyrene particles and to 1.1- μ m glass particles, these formulations did not result in increased immunogenicity compared to monomeric protein

controls. However, when rmGH was adsorbed to the same 1.1- μm glass particles, it showed a response that was as high as when the protein was adsorbed to aluminum hydroxide particles, a classical vaccine adjuvant.³² Also, when rmGH immunogenicity was tested with the same stainless steel microparticles used in the rhIFN β -1a study of van Beers et al., there was no enhancement of immunogenicity.³² The addition of aluminum hydroxide microparticles to rhIFN β -1a did not increase the protein's immunogenicity compared to that of the monomeric protein alone.⁶⁹ High numbers of IgG2-coated microspheres that were 5 μm in size were able to induce a low and transient ADA response in the Xeno-het mouse, in contrast to protein-coated nanospheres that did not induce a response.²¹ The immunogenicity of a murine anti-TNF α IgG2c/ κ antibody in mice was enhanced when the protein was adsorbed to the surfaces of micron-sized glass or aluminum hydroxide microparticles, or to the surface of micron-sized silicone oil droplets.²⁷ The antibody showed minimal conformational change upon adsorption to glass or aluminum hydroxide,⁴³ but was still immunogenic. Most recently, an emulsion of silicone oil droplets added to an ovalbumin formulation was shown to enhance immune responses to the adsorbed protein (in the absence of surfactant).²⁹ The different results obtained in these studies may be due to different mouse strains, molecule types, particle numbers, particle chemical compositions, extents of adsorption, reversibilities of adsorption and conformational changes, amongst other factors.

TREATMENT REGIMEN AFFECTS IMMUNOGENICITY

Dose and dosing schedule affect immunogenicity

A few studies in immune-tolerant mouse models have shown a positive correlation between aggregate dose and immunogenicity. Hermeling et al. observed an increase in anti-rhIFN α -2b IgG response with increasing aggregate dose, while the total rhIFN α -2b dose was kept constant.³⁶ More recently, in a study with rhIFN β -1b (Betaferon, a severely aggregated protein formulation⁵⁴) a clear trend towards higher IgG responses with an increasing total protein dose was seen.³⁰ In the latter study, an increase in number of doses or dosing frequency also led to higher anti-rhIFN β IgG levels, but one dose was found to be sufficient to break immune tolerance (for a discussion on immune tolerance and how it relates to natural abundance of proteins and other factors, please see^{44,70–72}). However, the type of aggregates and their chemical modification were not assessed in these studies. Stirring-induced aggregates of an IgG2 solution (containing $> 10^6$ micron-sized particles/ml, with most in the size range of 2–10 μm) were found to break tolerance in transgenic mice.²¹ However, a 100-fold dilution of these aggregates in the same total protein concentration (containing $> 10^4$ micron-sized particles/ml) did not break tolerance.⁷³ A similar result was obtained in a study using wild-type mice, where rmGH solutions containing microparticles (1.6 ng/dose, corresponding to $\sim 10^6$ micron-sized particles/ml, again with most in the size range of 2–10 μm) were immunogenic, but no immune response was detected when the particle concentration was reduced 100-fold to 0.02 ng/dose ($\sim 10^4$ particles/ml).³²

It has long been known that low or high doses of antigens may induce “low zone” or “high zone” tolerance, respectively.⁷⁴ These dose zones are dependent on the antigen in question. In a study using murine IgG, low doses of aggregated protein adsorbed to the surface of non-

proteinaceous microparticles or silicone oil droplets provoked stronger immune responses in mice than higher doses.²⁷ Similarly, repeated administration of high doses of IgG-coated microparticles resulted in significantly lower ADA responses as compared to low doses.²¹ These results highlight that it is challenging to choose an appropriate dosing schedule in murine models for evaluating immunogenicity of therapeutic proteins. One further complication is the difficulty in quantifying and comparing different dosing regimens, particularly when heterogeneous, aggregated material is used.

Administration route affects immunogenicity

The route and site of administration will affect the biodistribution of a therapeutic protein, its residence time, and the likelihood of encounter with antigen-presenting cells, and therefore its immunogenicity. Especially for injections into subcutaneous (SC) tissue, filtration effects may cause larger aggregates and particles (and some excipients) to concentrate in a small region at the site of injection.⁷⁵ In contrast, in intravenous (IV) administration, dilution effects may cause quick dispersion of impurities or degradants, as well as possible dissociation of aggregates and particles. It is not clear how these *in vivo* effects would scale from mice to humans, making it difficult to compare particle doses and effects of administration route. This question is further complicated by the fact that mice and humans exhibit major anatomical and physiological differences, particularly with respect to the structure of their skin and subcutaneous space. It has been stated that IV administration has a lower immunogenicity risk than SC administration,^{5,10} but in fact there are few preclinical and clinical data supporting this dogma.

The frequently-cited preclinical study on the effect of administration route on the immunogenicity of rhIFN α -2 α by Braun et al.⁴⁵ was performed in (non-immune-tolerant) wild-type mice rather than in immune-tolerant mice. Moreover, recently opposite results were observed in immune-tolerant mouse models, i.e., IV injected Betaferon was more immunogenic than SC, intramuscular or intraperitoneal injection³⁰ and IV administration of rmGH particles elicited higher IgG responses than the same formulation injected SC.²⁶

The aggregates associated with drug product administered IV may have a faster disposition than those delivered via the SC route. One study using whole mouse fluorescent imaging suggested that IgG1 aggregates could be retained at the SC injection site for over 1 month, thereby possibly increasing the chances for an immune response.⁷⁵ In another study, optical imaging in live mice was used to show that fluorescently tagged IgG2 can form high amounts of aggregate *in vivo* in the SC space, where it was retained for an extended period of time and could be taken up by immune cells.⁷⁶ However, this retention at the injection site did not cause significant ADA formation or major loss in exposure.⁷⁷

IMMUNE MECHANISMS ARE NOT YET FULLY UNDERSTOOD

An early hypothesis was that immune tolerance for recombinant human therapeutic proteins would be broken via a T-cell independent mechanism through the direct activation of B-cells recognizing repetitive epitopes^{78,79} presented on protein aggregates.^{34,36,58,62} Additionally, T-independent isotype switching has been reported in mice through concomitant Toll-like receptor and B-cell receptor activation.⁸⁰

Although we cannot rule out this mechanism as a contributor to the immune response elicited by protein aggregates, it has become clear that T-cell dependent mechanisms do play a role. For example, depletion of T-helper cells led to abolition of the immune response of immune-tolerant mice against both aggregated rhIFN β -1b⁴¹ and aggregated IgG1.²³ The occurrence of isotype switching in other immune-tolerant mouse models also provides evidence of the involvement of T-helper cells in the breaking of immune tolerance.^{24,26,28,32,43} Moreover, the timing of T-cell recruitment and the number of germinal centers formed after administration of aggregated rhIFN β -1b were almost identical in immune-tolerant and wild-type mice.⁶⁹ Nevertheless, there are several indications that the breaking of immune tolerance occurs via mechanisms that differ from a classical immune response against foreign antigens. Marginal zone B-cells, a B-cell subset involved in T-cell independent responses, were shown to play a role in antibody production against aggregated rhIFN β -1b in transgenic immune-tolerant mice, suggesting involvement of direct, T-cell independent B-cell activation.⁴¹ Van Beers et al. observed an apparent lack of immunological memory in transgenic immune-tolerant mice, but not in wild-type mice, against aggregated rhIFN β -1b³⁹ and very similar results were found for rhIFN α -2a.⁸¹ Interestingly, conjugation of rhIFN β -1a to the cholera toxin subunit B, a strong T-cell dependent adjuvant, increased the anti-rhIFN β antibody titers but did not trigger immunological memory.⁶⁹ A similar observation was made for mAb-based biologics where highly stressed forms of mAbs were able to impact T-cell driven responses.²¹ However, in this case the response was short-lived as evident from the low magnitude and transient ADA response in the human IgG2 tolerized mice.

CONCLUSIONS AND RECOMMENDATIONS

Conclusions

Mouse models have served as important tools to investigate the effect of product-related attributes on immunogenicity. By employing these models, some factors that increase the potential risk of immunogenicity have been identified. The presence of high numbers of aggregates, and in particular aggregates composed of proteins with extensive chemical modifications such as those induced by oxidation or photolysis, in association with limited loss of native conformation, seems to increase the relative risk of immunogenicity. However, the amount and type of aggregates in these studies were not necessarily representative of what is typically present in drug products. In some studies larger protein particles, up to about 10 micrometers in size, appear to be more immunogenic than small oligomers, but results are anecdotal and it has become clear that attributes other than size may be more important; more research is needed to rigorously establish the effect of aggregate size on immunogenicity risk. Furthermore, non-proteinaceous subvisible particles with adsorbed protein can sometimes lead to enhanced immunogenicity, although not consistently for all types of particles and all proteins. It is worth noting that since particle size is recognized as a contributor to immune responses to vaccines^{82–84}, it is reasonable to assume that size may also be an important attribute to consider in the context of immunogenicity of therapeutic protein aggregates and particles.

Although the level of risk to human patients cannot be defined from results with animal studies, the collective findings from these studies suggest that it is sensible to minimize levels of aggregates and particulate impurities in formulations of therapeutic proteins, as low as reasonably possible. In this context, one should keep in mind that improper storage, compounding or administration practices might lead to the formation of substantially increased numbers of degradants. Therefore, instructing end-users about proper handling of therapeutic protein products, as well as creating general awareness of the risk of improper handling, remains of utmost importance.

Recommendations for future studies

- *Test the immunogenicity of well-defined and characterized aggregates.* Protein aggregation can occur by many different pathways, resulting in a continuum of sizes from dimers to particles hundreds of micrometers in size and with many different characteristics.⁶⁶ Because of this, a thorough characterization of the aggregates should be included in any study on their ability to induce immunogenicity. Aggregates tested so far were mostly heterogeneous in size and other characteristics. Moreover, in several of the published studies comparing the immunogenicity of several stressed products, the products differed in aggregate content, size distribution and degree of chemical modification and conformational perturbation, making it impossible to pinpoint the factor(s) responsible for the different levels of immunogenicity. Therefore, it is recommended to test better defined aggregate populations which are as homogeneous as possible in terms of size, reversible versus irreversible association, chemical modifications, morphology, hydrophobicity, etc., as well as other types of degraded products.
- *Understand the similarities and differences between particles patients receive and those generated for immunological studies in model systems.* Currently, most of the testing in animal models has been with stress-induced aggregates generated specifically for the particular study. The aggregate size distributions, levels and aggregate types (e.g., reversible versus irreversible, heterogeneous versus homogeneous), and the conformational and chemical states of protein molecules within these aggregates (e.g., unfolded, partially unfolded, native-like; unmodified or chemically degraded) may be quite different than those that would be present in an average commercial product. Currently, it is not clear which of the observations made in model systems can be generalized and extrapolated to human beings. In addition, once a product has left the manufacturer it could be subjected to sub-optimal storage and handling conditions, and the aggregation profile could differ substantially from the state immediately upon lot release. Thus, characterization of aggregates in drug that patients actually receive, post lot-release, should also be undertaken. Until we understand how the different amounts and attributes of protein aggregate populations being tested relate to those actually injected into patients, it will be very difficult to determine the potential safety risk of the protein aggregates and particulates in drug products.

- *Test the impact of impurities other than aggregates.* While most of the studies so far have been devoted to the effect of aggregates on immunogenicity, very little is known about the effect of other impurities and contaminants, such as host cell proteins and DNA, and endotoxins, as well as the effect of sustained release formulations on immunogenicity. In some cases impurities may also lead to protein degradants that increase the potential immunogenicity of a biotherapeutic indirectly. For example, a study by Seidl et al.⁸⁵ suggested that tungsten-induced aggregation may relate to an increased immunogenicity potential for epoetin. In any case, studies of impurities or contaminants should always include a thorough assessment of the impact of the impurity on the protein active ingredient.⁸⁶ Immune-tolerant mouse models could serve to study these factors and further research into this is recommended.
- *Test the effect of administration route, particle concentrations and aggregate characteristics on biodistribution and immunogenicity.* The effect of the administration route, particle concentrations and aggregate characteristics, such as particle size distribution, on the disposition of aggregated proteins and immunogenicity risk requires more research. At the present time it is unknown how doses of aggregates and particles administered to mice should be scaled to afford better prediction of immune responses in humans. Compared to the doses of particles that humans might receive in a commercial product, what particle dose in a mouse would constitute a “high” “typical”, or “low” dose? Because local concentrations of particles in tissue and organs are likely to impact immunogenicity, the immunogenicity risk associated with route of administration needs to be evaluated in the context of drug disposition by both IV and SC tissue architectures, immune cell interactions in the SC and systemic space, and impact of excipients, drug formulations and devices that are used to facilitate biotherapeutic delivery. Furthermore, one should keep in mind that the immunogenicity of highly aggregated formulations might depend differently on the administration route than that of a product containing predominantly monomers, because of differences in biodistribution^{75,87} and potential susceptibility to further aggregation post injection.
- *Correlate preclinical immunogenicity data with clinical risk.* Models have been developed such that they are sensitive and discriminative; however, how an enhanced immune response relates to clinically relevant risk is yet unknown. Data collected during clinical trials and during post-marketing surveillance could be used to attempt to correlate immunogenicity in patients with attributes of a particular drug product. This information could then be compared to results in mouse models to understand the clinical relevance of these model systems. These studies can however be very difficult to interpret for a variety of reasons, including the fact that the human patient population is genetically heterogeneous, and individual patients could have been on other treatments previously, etc.
- *Make better use of immune-tolerant mouse models by expanding read-outs.* Up until now, the read-out for immunogenicity in immune-tolerant mouse models has been primarily antibody formation. However, antibodies are just an end-product of an evolving immune response. Measurement of innate and adaptive immune

responses, antigen localization, allergic and hypersensitivity reactions, trafficking, and residence time should be considered to gain more insight into the mechanisms leading to an immune response.

- *Develop new or improved immunogenicity models.* Studies to date have demonstrated large strain-dependent differences in murine immune responses to therapeutic proteins; additional studies are needed to better understand these strain dependent effects. At present, it is not understood how to choose or develop a mouse strain that optimally mimics human immune responses. One avenue for development of improved mouse models is the use of humanized mice, which are translational models for studying human immune responses, especially where there is lack of an appropriate knock-out model. Immune deficient mice can be humanized either with human hematopoietic stem cells or peripheral blood mononuclear cells, thereby creating appropriate model systems to understand association of human HLA alleles in diseases such as allergy and autoimmunity, and to address specific questions related to immune responses to biologics and their attributes.⁵¹ These mice could also facilitate the identification of peptides presented by HLA-DR molecules from an antigen in question, especially from human biologics or recombinant proteins. Hence, such transgenic mice have provided value in understanding the pathogenesis of disease and can identify antigenic sequences from a biologic under evaluation that could predispose a subject to a higher immunogenicity risk. Reconstituted “humanized” mice treated with anti-CTLA-4 antibody (ipilimumab) were first used to understand the pathology of an autoimmune response followed by treatment with another biologic; to evaluate the efficacy and improvement in disease.⁸⁸ Creation of double transgenic mouse models expressing the human protein of interest and human HLA or human hematopoietic bone marrow, thymus and liver is another approach that could be explored. These can provide a better understanding of immune tolerance and long term memory response to biologics and factors that can disrupt such tolerance. Other mouse models that have not yet been used for immunogenicity studies, but might be useful, are included listed in Table 1. Perhaps making a better use of other animal models (e.g., pigs, minipigs^{89,90}) in the future may help circumvent some of the anatomical and physiological differences between mouse and human which complicate current studies.
- *Combine immune-tolerant mouse models and other preclinical models.* Very few studies have been performed combining the results of different preclinical models on the same samples. For instance, systematic studies on samples enriched in, e.g., dimers, oligomers, sub-micron aggregates and micron-sized aggregates by using *in vitro* and *in vivo* models in parallel would not only provide insight into the relative sensitivity of the different models but also teach us about the relationship between innate and adaptive immune responses.

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Table 1

Advantages and limitations of immune-tolerant mouse models.

Mouse model	Purpose	Advantages	Limitations	Key references
Wildtype mice	Evaluate murine variants of human proteins	Relevant model with murine immune system evaluating murine variant-induced tolerance Evaluate the effects of different product quality aspects, e.g., impurities or conformation	Murine proteins are not necessarily predictive of human proteins and their binding to relevant target Impurity profiles, potentially affecting immunogenicity, may differ between commercial proteins and their murine counterparts	26-28,42,43,65
Transgenic mice	Tolerized to a human protein	Mimic human subjects that are born with a specific tolerance to an endogenous protein	Labor intensive Each protein that needs evaluation requires a new transgenic mouse to be bred Need additional characterization for confirming the presence of transgene Use murine immune system	21,23,25,34,35, 38,45,46
XenoMouse	Evaluate attribute related risks to human IgG2	Tolerant to human IgG Can respond to neo-antigens	Weak B cell signaling Unable to mount robust immune response to molecules with low immunogenic potential May not be sufficiently robust and sensitive to detect small changes in attributes	22,47
Xeno- heterozygous (Xeno-het) mouse	Evaluate attribute related risks to human IgG2	Tolerant to human IgG Immune robust and can respond to neo-antigens	Inadequate B cell signaling Relies on murine T cells and antigen- presenting cells;	21
Severe combined immune deficient mice (SCID) mice Human peripheral blood (PBL) grafted SCID mice (SCR)-SCID mice (hematopoietic SCID- replacing cells)	Can be used to study viral antigen specific responses Good model for studying human infectious diseases and test the efficacy of antimicrobials	Engraftment of human self-renewing hematopoietic progenitors High level reconstitution with human immune cells	Engraftment is highly variable Injected human progenitor cells rarely gave rise to all hematopoietic-derived lineages Intrinsic limitations of the murine recipient Development of human T cells in murine thymus is suboptimal and delayed	51,91-93
BLT (Bone marrow hematopoietic stem cells/ human liver and thymus tissues)	Can be used to study human immune response especially the human T cell response	Robust immune repertoire, consistent engraftment of human cells, including multiple hematopoietic lineages, T cells educated on human thymic epithelium	Lack of HLA molecules for T cell education	94
MHC class I and II double-knockout mice crossed with the Hu-SRC- SCID or BLT models	Study sequence-based immunogenicity risk and MHC restricted antigen presentation Serve as <i>in vivo</i> models for the prediction of the immunogenicity of humanized and/or fully human biologics	Human T-cell restriction by a human HLA Robust long lived human immune cell repertoire	Not tolerant for all human proteins Cumbersome as each mouse has to be engrafted due to no germline transfer of genes encoding human immune cells Variability in immune responses to neo- antigens	94
Xeno-het mouse crossed with the MHC class I and II double-knockout mice or the Hu- SRC- SCID or BLT models	Good models for studying immunogenicity risk due to human biologics Can be used to evaluate sequence based risk and immune tolerance	Human T-cell restriction by a human HLA Robust long lived human immune cell repertoire Human IgG tolerant	Not known; still to be evaluated	Not available